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MINI REVIEW

Snake venom α-neurotoxins and other 'three-finger' proteins

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The review is mainly devoted to snake venom α-neurotoxins which target different muscle-type and neuronal nicotinic acetylcholine receptors. The primary and spatial structures of other snake venom proteins as well as mammalian proteins of the Ly-6 family, which structurally resemble the 'three-finger' snake proteins, are also briefly discussed. The main emphasis is placed on recent data characterizing the α-neurotoxin interactions with nicotinic acetylcholine receptors.

Keywords: acetylcholine receptors; binding surfaces; neurotoxins; primary structure; spatial structure; 'three-finger' proteins.

About 30 years ago, α-bungarotoxin and other α-neurotoxins from the venoms of *Elapidae* and *Hydrophidae* snakes (*Bungarus*, *Naja* and other species) made possible identification and isolation, from electric eels and rays, of the nicotinic acetylcholine receptor (AChR), the first receptor protein subsequently characterized in detail (reviewed in [1–3]). At present, with the molecular biology methods available, the toxins are less important when researchers are interested in finding and sequencing new receptors. However, receptors belonging to the same family or superfamily might have different pharmacological specificities, which is of prime importance for understanding their function under normal conditions and in pathologies. In this respect, it is an ever expanding arsenal of neurotoxins available to researchers that helps distinguish even subtle pharmacological differences in AChRs.

Compared with cloning, progress in determining the spatial structure of receptors and ion channels is much slower. On the other hand, X-ray and NMR solution structures have been elucidated for many peptide and protein neurotoxins. In combination with experimental studies on the receptor–neurotoxin interactions, spatial structures of neurotoxins can shed light on the three-dimensional organization of the respective binding sites in receptors.

PRIMARY STRUCTURE OF THE 'THREE-FINGER' PROTEINS

α-Neurotoxins from snake venoms that potently block nicotinic acetylcholine receptors contain 60–75 amino acid residues and are fixed by 4–5 disulfide bridges (Fig. 1). The major difference between the short-type (four disulfides, 60–62 residues) and long-type (five disulfides, 66–75 residues) α-neurotoxins, which have comparable affinity for the *Torpedo* and muscle-type AChRs, was known to be in the kinetics of association/dissociation with the receptor. However, as found recently, only long-chain neurotoxins block potently α7 homo-oligomeric neuronal AChRs. It has also been shown that the integrity of the additional disulfide bridge in the central loop II of neurotoxins (Fig. 2) is essential for high-affinity binding to α7 AChR [4]. The removal of the disulfide bond Cys27–Cys31 in κ-bungarotoxin ('neuronal bungarotoxin') selective for neuronal AChRs composed of α3 and β2 subunits also brings about a considerable drop in the affinity for the receptor [5].

The venoms of different snakes contain small proteins that have a similar size and a homologous disposition of disulfide bridges as α-neurotoxins. The three disulfide-confined loops (Fig. 2) have a characteristic 'three-finger' appearance. Among these proteins are several muscarinic toxins which differ in selectivity towards distinct subtypes of muscarinic acetylcholine receptors [6], fasciculins that inhibit acetylcholinesterase [7], calciseptins and other toxins that block the L-type Ca^{2+} channels [8,9]. Cardiotoxins (or cytotoxins) apparently do not have a specific receptor or ion channel target, rather they appear form channels in membranes [10]. Dendroaspins (mambins) are distinct from all other toxins by having an RGD triad in the loop III, and as a result they act as antagonists of various cell adhesion processes [11].

Figure 1 shows several amino acid sequences representing the three-finger proteins of different classes. Interestingly, proteins from various snake species form one of the most abundant families of homologous proteins (over 150 proteins sequenced), and in recent years new toxins or their isoforms have been discovered by cDNA analysis (e.g. [12]).

In the lower part of Fig. 1 there are sequences of proteins which are not toxins, but have a striking resemblance to the three-finger toxins. These proteins belong to the Ly-6 superfamily of mammalian cell-surface molecules anchored to the cell via a glycophosphatidylinositol tail [13–15]. The Ly-6 proteins contain an additional disulfide in the loop 1, which is also present in the so-called 'weak' toxins [16]. Most proteins of Ly-6 family contain one toxin-resembling domain of 70–90 amino acid residues. The uPAR (urokinase plasminogen activator receptor) is much larger and has even three such 'toxin-looking'domains [15].

Another small group of proteins that in their chain length and disposition of cysteines have similarity to cardiotoxins and

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Abbreviations: AChR, nicotinic acetylcholine receptor.

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        1         10        20        30        40        50        60
Erb     RICFNHQSSQPQTTKT----CSPGESSCYHKQWSD-F---RGTIIERG-CG----CPTVK-PGIKLS-------CCESEV-CNN
α-BTX   IVCH-TTATIPSSAVT----CPPGENLCYRKMWCDAFCSSRGKVVELG-CAAT--CPSKK-PYEEVT-------CCSTDK-CNHPPKRQPG
κ-BTX   RTCLISPSSTPQT-------CPNGQDICFLKAQCDKFCSIRGPVIEQG-CVAT--CPQFRSNYRSLL-------CCTTDN-CNH
CT      LKCKKLVPLFS---KT----CPAGKNLCYKMFMVAAP----HVPVKRG-CIDV--CPKSSLLVKYV--------CCNTDK-CN
Fas     TICYSHTTTSRAILKD----CGENS--CYRKS-RR-HPP-K-MVLGRG-CG----CPPGD-DYLEVK-------CCTSPDKCNY
CLS     RICYIHKASLPRATLT----CVENT--CYKMF-IR-TQR-E-YISERG-CG----CPTAMWPYQTE--------CCKGDR-CNK
MTX2    LTCVTTKSIGGVTTED----CPAGQNVCFKRWHYVTPKNYDIIKG----CAAT--CPKVDNNDPIR--------CCGTDK-CND

Xen     LKCVNLQANGIKMTQE----CAKEDTKCLTLRSLK--------KTLKF-CASGRTCTTMKIMSLP----GEQITCCEG-NMCN
CD59    LQCYNCPN--PTADCKTAVNCSSDFDACLITKA-------GLQVYNK--CWKFEHCNFNDVTTRLRENEL--YYCCKKDL-CN
uPAR-1  LRCMQCKT---NGDCR-VEECALGQDLCRTTIVRLWEE-GEELELVEKSCTHSEKTNRTLSYRTGLKITSLTEVVCGLDL-CN
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Fig. 1. Representative amino acid sequences of the 'three-finger' proteins. Erb, erabutoxin *b Laticauda semifusciata*, a short-type α-neurotoxin; α-BTX and κ-BTX, α- and κ-bungarotoxins from *Bungarus multicinctus*; CT, cytotoxin II from *Naja oxiana* (see references for these three toxins in the review [1]); MTX2, muscarinic toxin from the green mamba *Dendroaspis angusticeps* [6]; Fas, fasciculin from *Dendroaspis angusticeps* [7]; CLS, calciseptine from the black mamba *Dendroaspis polylepis*; CD59, human complement regulatory protein CD59 [15]; uPAR-1, urokinase plasminogen activator receptor [15] (for the latter two proteins only partial N-terminal sequences are given); Xen, xenoxin from *Xenopus laevis* [17].

short-chain neurotoxins, are xenoxins. These proteins, whose function is still unknown, were isolated from the skin secretion of the *Xenopus laevis* frogs [17].

SPATIAL STRUCTURE OF 'THREE-FINGER' PROTEINS

The first X-ray structure of a three-finger toxin was that of erabutoxin *b* determined about 20 years ago [18,19]. Subsequent NMR and X-ray studies on various short- and long-chain α-neurotoxins revealed that these proteins have a similar conformation both in the crystal and in solution.

The characteristic feature of all α-neurotoxins is the disposition of four disulfide bridges forming a hydrophobic core. From this core protrude three disulfide-confined loops ('fingers') I, II, III (Fig. 2). There are practically no α-helical segments in the neurotoxins, while β-structure amounts to about 40%. A prominent feature of the α-neurotoxin conformation is a triple-stranded β-structure formed by the two segments of the central loop II and by the fragment of loop III.

Interestingly, a similar topology was revealed in all other 'three-finger' proteins, as can be seen from Fig. 2. Even in the case of the 'nontoxin' CD59 protein [14], the topology is very similar: a short turn of α-helix in the loop III brought about no big differences. An extra disulfide in loop I also does not destroy the kinship.

As mentioned above, the short- and long-chain α-neurotoxins have some differences in the kinetics of association/dissociation with the *Torpedo* receptor, which are difficult to ascribe to any features of the three-dimensional structure, associated with either the presence of the C-terminal tail in the long-chain toxins or with their additional disulfide in the loop II. Interestingly, the conformational role of the latter became clear from the comparison of the three-dimensional structures of α-neurotoxins and α-conotoxins, oligopeptide neurotoxins from the poisonous marine snails of the genus *Conus*. Before the X-ray or NMR structures of any α-conotoxins were known, it was hypothesized that there would be a similarity in spatial organization of such α-conotoxins as G1 or M1 (acting on *Torpedo* and muscle-type AChRs) and the loop II of the short-chain neurotoxins, especially when the respective sequences are compared in opposite directions [25,26]. However, the recently determined X-ray [27] and NMR [28,29] structures of α-conotoxin G1 (Fig. 3) revealed in the major conformer an α-helical turn Gly8–Tyr11 which does not have a direct analogy with the tip of loop II in short α-neurotoxins (Fig. 2, NT-II structure). On the other hand, the NMR structure of α-conotoxin ImI [30], acting on neuronal α7 AChRs, does unveil a common scaffold (in the region which includes a functionally essential triad DPR [31]) with a helix-like tip of the central loop of the long-chain α-neurotoxins (Figs 2 and 3). It means that the conformation of the tip ensured by the additional disulfide is an essential determinant for interacting with neuronal AChRs [30]. Interestingly, α-conotoxin G1 is not only less similar to α-cobratoxin, than α-conotoxin ImI, in the backbone folding, but also lacks a pair Trp10/Arg7 of ImI resembling in its disposition the Phe29/Arg33 of α-cobratoxin.

What structural features distinguish one particular three-finger toxin from its relatives? Due to insertions or deletions, the size of some loops may differ; certain loops may be closer to each other, reflecting the tightness of the interloop interactions. There may be differences in primary structure (for example, a high content of positively charged residues in cardiotoxins), which can affect the character of molecular surface. Marked differences in the overall stability, as well as in the flexibility of the specified loops (e.g. [32]), were detected between toxins belonging to the same or different groups.

What allows one to distinguish between different classes of toxins, is the tendency of some to oligomerize. In contrast to essentially monomeric α-neurotoxins, κ-bungarotoxin exists exclusively as a dimer, both in the crystal and in solution [23,33]. For cardiotoxins, X-ray analysis revealed trimers, with a hydrophobic exterior and a hydrophilic pore in the middle. It gave rise to the hypothesis that various effects of cardiotoxins may be realized via formation of such pores or channels in membranes [10].

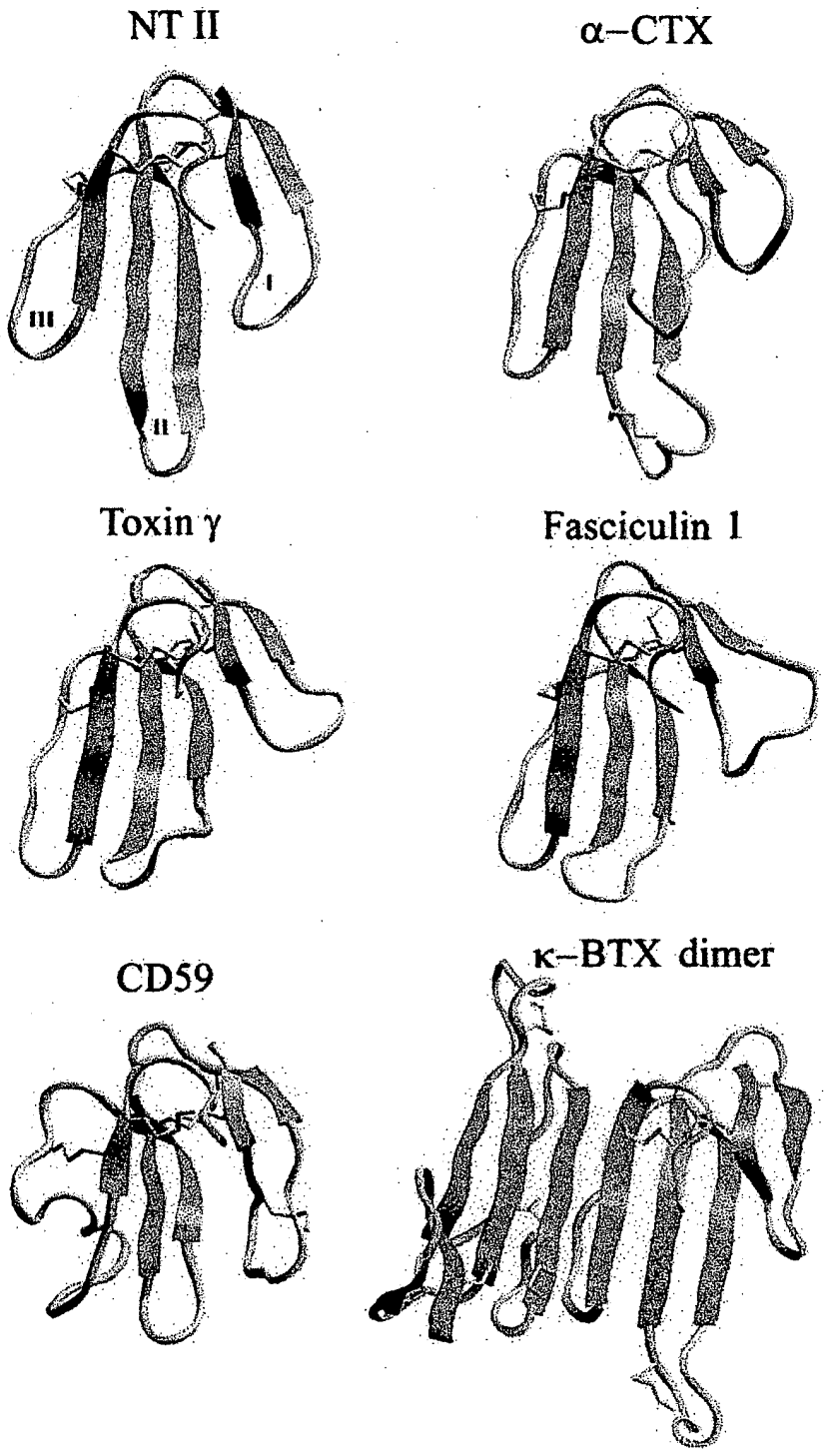

Fig. 2. Spatial structures of 'three-finger' proteins. Protein Database accession numbers are given in parentheses. NT II, neurotoxin II *Naja oxiana*, NMR structure [20] (1NOR); α-CTX, cobratoxin *Naja siamensis*, X-ray structure [21] (2CTX); Toxin γ, cytotoxin (cardiotoxin) from *Naja nigricollis* venom, X-ray structure [10] (2CTX); Fasciculin 1, X-ray structure [22] (1FAS); κ-BTX, κ-bungarotoxin dimer, X-ray structure [23] (1KBA). The disulfide-confined loops are marked as I, II, III in the NT II structure. All other proteins are shown in similar orientation, that is with the N-terminal loop I to the right from the central loop II. Blue ribbons depict the β-structures, disulfide bridges are shown in light yellow. The structures are presented with the aid of program MOLMOL [24].

SPATIAL ASPECTS OF INTERACTION BETWEEN THREE-FINGER PROTEINS AND THEIR TARGETS

In this section, the focus will be mainly on α-neurotoxin binding to AChRs. Many laboratories applied different approaches to outline the interacting surfaces of α-neurotoxins and the receptor. Since most of the invariant functional groups are concentrated in the loop II, after first X-ray analyses it was ascribed a major or even an exclusive role in binding to AChR. However, studies on the *Torpedo* AChR interaction with a series of neurotoxin II derivatives containing spin, fluorescence, or photoactivatable labels provided experimental support for the multiattachment mode of the neurotoxin binding and demonstrated that the loops I, II, and III can contact the receptor [34–37]. Recently, extensive mutagenesis of erabutoxin *a*

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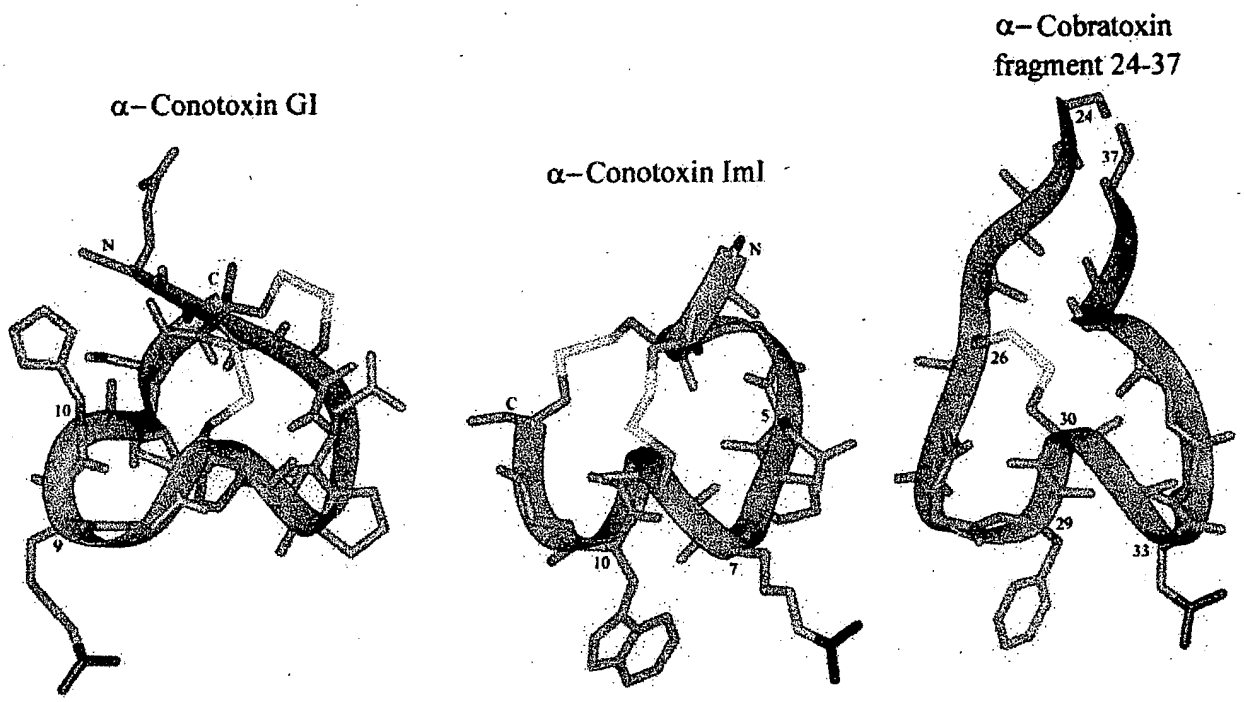

Fig. 3. Comparison of three-dimensional structures of α-conotoxins G1 and ImI with the central loop of α-cobratoxin. Protein Database accession numbers are given in parentheses. α-Conotoxin G1 from *Conus geographus* marine snail, major conformation, NMR structure [28]; α-conotoxin ImI from *Conus imperialis*, NMR structure [30] (1ImI); α-cobratoxin fragment 24–37, X-ray structure [21] (2CTX). Blue ribbons depict the polypeptide backbones, disulfides are shown in yellow. The Arg and aromatic-residue side chains discussed in the text are shown in dark blue and green, respectively.

demonstrated an important role for some specific residues in loops I and II, with some contributions to binding also coming from loop III [38].

Until recently, data concerning the toxin-binding surface in the receptor were mainly on the role of amino acid residues within the α-subunit fragment 180–200. For example, the resistance of the snakes and mongoose against α-bungarotoxin was found to be the result of substitutions in this region of their AChRs [39]. In addition, introduction of glycosylation sites to this fragment made AChRs of rat muscle less sensitive to snake venom α-neurotoxins [40].

On the other hand, crosslinking studies with photoactivatable derivatives of snake venom α-neurotoxins revealed contacts to both α and other 'nonα' subunits of the receptor (references in [2]). The binding sites for snake venom α-neurotoxins are situated at the interfaces between α/γ and α/δ subunits [37], in a manner similar to those for d-tubocurarine [41], α-conotoxins from marine snails [40,42,43], or waglerins (22-residue peptides from South-east Asian snake *Trimeresums wagleri*) [44]. However, in contrast to other toxins, α-neurotoxins do not show marked differences in the affinity for the two binding sites. Interestingly, such differences appear when certain changes are introduced into the neurotoxin structure, such as acylation of all amino groups in neurotoxin II *Naja oxiana* [34] or changing the positive charges of Lys27 or Arg33 for negatives ones (K27E or R33E mutations) in α-neurotoxin *Naja mossambica* [45].

In contrast to many of the residues identified as important for binding low molecular mass agonists or antagonists (reviewed in [2]), there is much less data on the receptor residues directly involved in the interaction with snake α-neurotoxins, or located in the vicinity of binding sites. A photolabel attached via a spacer to Lys25 of neurotoxin II *Naja oxiana* generated a photoinduced crosslink with Ala268 of the δ subunit, which gave an estimate of the neurotoxin distance from the lipid bilayer [46]. There is a preliminary report [47] that a maleimido group introduced at position 33 of the mutated α-toxin *Naja nigricollis* forms a covalent bond with the reduced disulfide of Cys192-Cys193 of the α-subunit. Using double mutant cycles to identify pairwise interactions, it was found that invariant Arg33 of the *Naja mossambica* α-neurotoxin is close to Val188 of the α-subunit of the mouse muscle AChR [48].

Until very recently, the functional role of α-neurotoxin contacts with 'nonα' subunits was not clear. However, Sine [49] demonstrated that mutations of γLeu119 and δLeu121 drastically decreased the binding of α-bungarotoxin to the oligomeric receptor complex.

Although there is fragmentary data, either from crosslinking or mutagenesis, we are still lacking a high resolution X-ray structure which would directly demonstrate which groups of the α-neurotoxin molecule interact with the identified amino acid residues of the intact nicotinic acetylcholine receptor. As a first approximation to this goal, one can consider the NMR structures of the α-bungarotoxin complex with the synthetic peptide KHWVYYTCCPDT corresponding to the *Torpedo* α-subunit fragment 185–196 [50], or the complex with a peptide MRYYESSLKSYDD from a combinatorial library [51]. In the first case, the authors describe binding of the receptor peptide in the 'arm pit' of α-bungarotoxin created by the loops I and II, and His68 of the C-terminal tail. The library peptide binds in a similar region (a 'pocket') of α-bungarotoxin. However, in contrast to the receptor fragment, which has an extended conformation, the library peptide is almost a globular shape [51]. Quite clearly, the contributions of the whole oligomeric AChR into toxin binding cannot be reduced to the interactions provided by a relatively short peptide sequence. First, the role of other subunits is ignored. Second,

even within the α-subunit itself, a number of residues outside the 185–200 fragment, such as Tyr93, Trp149, and Asp152 in *Torpedo* and muscle receptors [2,52], Trp55 and Thr77 in α7 AChR [53] were found to be involved in binding of low molecular mass ligands, and one might expect that similar sites are involved in binding of larger molecules, such as snake α-neurotoxins. It seems more appropriate to use the whole N-terminal domain of the α-subunit for structural studies on α-neurotoxin complexes. This domain (amino acid residues 1–209) was recently heterologously expressed by several groups as a protein suitable for functional and structural analyses [54–57].

Historically, with the *Torpedo* acetylcholine receptor, polypeptide neurotoxins were for the first time used for comprehensive studies on the receptor organization. However, this hetero-oligomeric ion channel appeared to be too complicated to utilize the whole potential of toxins. More rapid progress has been achieved with 'simpler' targets of polypeptide toxins. In 1995, two groups determined the high-resolution X-ray structures of fasciculin bound to acetylcholinesterase [58,59]. In the complex, the toxin essentially preserved its shape. The main contributions to binding were from the loops I and II of the toxin. Another characteristic feature was a multipoint attachment of the toxin to the enzyme. As mentioned above, a similar mode of binding has been earlier deduced for snake venom α-neurotoxins from affinity labeling [37] and mutagenesis data [38].

A multisite attachment to their target is also a property of polypeptide neurotoxins from scorpion venoms. These neurotoxins do not belong to the 'three-finger' proteins, but are comparable to the latter in size. Recent X-ray and mutagenesis data [60] suggest that such proteins as charybdotoxins penetrate relatively deeply into potassium channels. In the light of this finding, the idea of snake venom α-neurotoxins binding in a gorge between the AChR subunits [2] might underlie a common mechanism of interaction between polypeptide neurotoxins and ion channels.

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